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Interspecific Relationship and Ecological Requirements of Two Potentially Harmful Cyanobacteria in a Deep South-Alpine Lake (L. Iseo, I)

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Abstract: In Lake Iseo (Lombardia, Italy), the predominant species in the cyanobacterial taxa was *Planktothrix rubescens*. However, since 2014, the presence of an allochthonous Cyanobacteria, *Tychonema bourrellyi*, able to produce consistent biomasses and harmful toxins, was detected. The causes of this expansion are poorly understood. Many studies have linked the development of allochthonous Cyanobacteria populations with climate change. This study shows the spatio-temporal dynamics, the ecological requirements, and the interspecific relationship of *P. rubescens* and *T. bourrellyi*. Samples were collected monthly in 2016 at six different depths in the water column; 20 chemico-physical characteristics were measured; and Cyanobacteria density, morphology, and biovolume were evaluated. The results allowed a comparison of the spatial pattern of the two species, which showed a greater distribution at a depth of 10–20 m, and their seasonal dynamics. Both Cyanobacteria were present throughout the year, with the greatest abundance during the period from March to May. A temporal shift was observed in their development, linked to different capacities for overcoming winter and mixing periods. Principal Component Analysis, performed on 20 observations (4 months × 5 depths), highlighted the important role of the stability of the water column in determining *T. bourrellyi* settlement in Lake Iseo and the role of solar radiation in spring population development.

Keywords: Cyanobacteria; colonization; *Tychonema bourrellyi*; *Planktothrix rubescens*; deep lake; stability; PCA

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1. Introduction

Cyanobacteria are the most ancient phototrophs on the earth. As a result of their long evolutionary history (~2 by) [1,2], and due to their high ecological plasticity, Cyanobacteria are able to adapt to geochemical and climatic changes [3,4]. Consequently, these organisms are also capable of adapting to anthropogenic modifications of aquatic environments [5], such as enhanced nutrient loading or increasing temperatures. The literature has reported that climate change is likely to stimulate the development of harmful Cyanobacteria (e.g., [6–14]). Regional and global warming, with an associated increase in temperature and variability in rainfall patterns, causes changes in nutrients, sediment delivery, sediment-water exchange and metabolism, water residence time, and vertical stratification [6,12]. Therefore, ecosystems are subjected to alterations that affect the biotic community, modifying the species composition with the invasion and the establishment of allochthonous populations of Cyanobacteria in freshwater ecosystems [4,15,16].

Increasing focus is being directed towards harmful Cyanobacteria in aquatic ecosystems, as these organisms are capable of forming consistent blooms (cyanoHABs) affecting use, safety, and sustainability of water resources, with considerable ecological and socioeconomic costs [1,17,18]. Cyanobacteria blooms represent an appreciable threat not only to human health through exposure to toxic compounds, such as via drinking water, aquaculture, and recreation [18,19], but also for the biological community, because the proliferation of these organisms can affect the functioning of the ecosystem [11,17]. Nevertheless, the impact of cyanotoxins on aquatic ecosystems remains poorly understood [20], so acquiring more information about the toxicological features and the autoecology of the species able to produce cyanotoxins is important. These information would help increase understanding of the environmental drivers that promote the proliferation of harmful Cyanobacteria, and consequently provide further information for water managers and policy makers.

Studies on the phytoplanktonic community and Cyanobacteria species have been performed for over 30 years in the great south-Alpine lakes in Italy (i.e., L. Maggiore, L. Como, L. Iseo, L. Garda) ([21–28]). Starting in 2014, the colonization and the development of *Tychonema bourrellyi* (J. W. G. Lund) [29], a Cyanobacteria able to produce anatoxin-A (ATX), were documented [3,4]. The extent and the causes of the expansion of this species in deep holo-oligomictic lakes (i.e., large lakes south of the Alps) remain to be explored [4]; information about its ecology and physiology are limited [30].

The aims of this paper were to understand how this allochthonous species affects the current cyanobacterial community, to determine which environmental factors favored the invasion, and to investigate the direct or indirect relationships between environmental changes (i.e., climate) and the development of potentially harmful Cyanobacteria. These goals were achieved by analyzing phytoplanktonic populations in Lake Iseo, a south-Alpine deep lake, in which one of the predominant Oscillatoriales over the past 15 years was *Planktothrix rubescens* (De Candolle ex Gomont) [3,22,23,29,31]. The main objectives were: (1) to highlight and compare spatio-temporal population dynamics of *P. rubescens* and *T. bourrellyi*, analyzing their maximum and minimum seasonal abundance, along with their distribution along the water column, to determine if the presence of an allochthonous species could modify the dynamics of the preexisting cyanobacterial community; (2) to evaluate lacustrine chemico-physical parameters that could affect their development. We supposed that the environmental changes that occurred in recent decades in Lake Iseo, driven by a climatic force acting at a regional scale [32–34], affected the phytoplanktonic community. In particular, the documented increases in water temperature and water column stability [35] could promote the colonization and development of *T. bourrellyi* in Lake Iseo.

2. Materials and Methods

2.1. Study Area

Lake Iseo is a deep perialpine lake, the fifth-largest Italian lake in terms of volume (7.6 km^3). The surface area is 61.8 km^2, the maximum depth is 258 m, and the mean depth is 124 m [22,36,37]. The lake, which is included in the LTER network (Site LTER_EU_IT_008—"Southern Alpine Lakes"; http://www.lter-europe.net), is situated in Northern Italy at the end of a prealpine valley, Val Camonica (Figure 1).

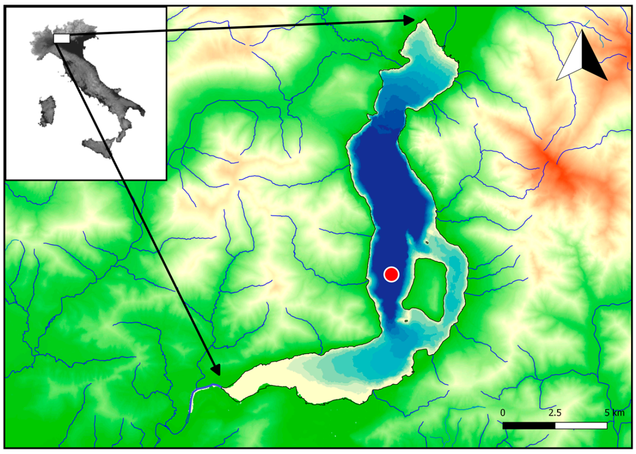

Figure 1. Location and bathymetry of Lake Iseo. The red dot indicates the position of the sampling point (modified [38]).

Lake Iseo should be classified as "warm monomictic", since winter water temperatures never drop below 4 °C. However, due to the great depth of the lake, winter mixing occurs only during cold and windy winters. Over the last twenty years, complete winter mixing occurred only in 2005 and 2006, so the lake can now be regarded as holo-oligomictic [21,22,36,39,40]. Except for these two years, the spring mixing depth from 1998 to 2016 in Lake Iseo ranged between 30 and 150 m, and in 2016 it reached about 50 m [35,36]. The extent of the mixing depth was determined by analyzing the homogeneity of the depth profiles of physical and chemical data recorded along the water column [35].

Nowadays, the increase in nutrient loading has brought Lake Iseo to a meso-eutrophic condition, with an average concentration of total phosphorus of around 80–90 µg/L [21,22,36].

2.2. Sampling, Field Measurements, and Laboratory Analyses

Samplings for the analysis of the two Cyanobacteria were carried out in Lake Iseo from January to December 2016. Samples were collected at the deepest point of the lake (45°43′11″ N; 10°03′46″ E) [22], at an almost monthly frequency, using a Niskin bottle, along the water column at six different depths (0, 10, 20, 30, 40, 60 m), collecting over all 72 samples. These depths were chosen in consideration of the extent of the euphotic zone in Lake Iseo.

An algae count was performed on fresh material, due to the difficulty of discriminating the filaments of *T. bourrellyi* and *P. rubescens* in samples fixed with Lugol solution [4]. From each sample, 10 mL was vacuum filtered onto 5 µm pore-sized Whatman Cyclopore transparent polycarbonate membrane filters (25 mm filter diameter) [4,41]. Filtration was made at low pressure to avoid damaging the filaments [4]. The filters were transferred on a microscopic slide and, employing a microscope (100× magnification), discrimination and quantification of the two species were carried out, analyzing the filaments in the whole area of the filters. The average length and average width of the cells were obtained by measuring at least 20 randomly selected sample filaments. These parameters allowed the estimation of their density (cell/L) and biovolume (mm^3/m^3), assuming cylindrical cell shape for both the taxa [42,43].

In the laboratory, determinations of total phosphorus, soluble reactive phosphorus, total nitrogen, nitrate nitrogen, ammonium nitrogen, conductivity, and alkalinity were carried out using standard methods, in accordance with APHA-AWWA-WEF [44]. Dissolved oxygen concentration, pH, and temperature were, however, detected in situ, instead, with a portable underwater multiparameter probe (WTW multi3432). Cations and anions were measured using the Ion Chromatography (Thermo Scientific^{TM} Dionex^{TM}, Waltham, MA, USA).

The water transparency (z_s) was detected monthly using the Secchi disk, and the depth of the euphotic zone (z_{eu}) was estimated as $z_{eu} = 4.8 \cdot z_s^{0.68}$ [45]. The thermocline depth was calculated monthly using the package 'rLakeAnalyzer' in R 3.4.1. [46], where the minimum density gradient was set by default to 0.1 kg/m^3/m. The penetration of solar radiation into the water column was obtained from the values of superficial global radiation (W/m^2) provided by ARPA Lombardia, with measurements made at the meteorological station of Costa Volpino (192 m a.s.l.), located ca. 1 km away from the northern border of the lake. Stability was, moreover, calculated monthly as the Brunt-Väisälä frequency (s^{-2}), using the package 'rLakeAnalyzer' [46,47].

2.3. Statistical Analyses

In order to highlight the ecological requirements of *P. rubescens* and *T. bourrellyi*, the relationship among selected chemico-physical parameters and biovolume of the two Cyanobacteria was analyzed using Principal Component Analysis (PCA) based on the correlation matrix [48,49].

Twenty chemico-physical parameters were selected for the PCA: water temperature "WT" (°C), water density "WD" (kg/m^3), pH, alkalinity "ALK" (meq/L), electrical conductivity "EC" (µS/cm), total phosphorus "TP" (µg/L), total nitrogen "TN" (µg/L), TP:TN ratio "TP.TN", soluble reactive phosphorus "SRP" (µg/L), ammonium nitrogen "NH4" (µg/L), nitrate nitrogen "NO3" (µg/L), silicates "Si" (µg/L), chloride "Cl" (mg/L), sulphates "SO4" (mg/L), calcium "Ca" (mg/L), magnesium "Mg" (mg/L), sodium "Na" (mg/L), potassium "K" (mg/L), light radiation "RAD" (W/m), and Brunt-Väisälä frequency "STABILITY" (s^{-2}).

All data were centered (mean value = 0) and scaled (variance = 1) to allow comparison among parameters. The normality distribution was checked using the Shapiro-Wilk test [50], and variables without a normal distribution were $\log_{10}(x + 1)$ transformed. The species biovolumes were Hellinger-transformed, in order to reduce the importance of large abundances [49,51,52].

A preliminary principal component analysis was performed, including all 72 observations, in order to highlight the most relevant parameters to focus on. A final PCA was performed considering only the months and the depths possessing the strongest relationships between the Cyanobacteria and the environmental features. The more relevant relationships between *T. bourrellyi* and chemico-physical parameters, showed by PCA, were tested with a linear regression analysis. All statistical analyses were performed by different packages in R 3.4.1. [53].

3. Results

3.1. Thermal Regime and Water Stability

From January to December 2016, temperature values in the first 60 m of the water column of Lake Iseo were between 6.5 and 23.8 °C (Figure 2).

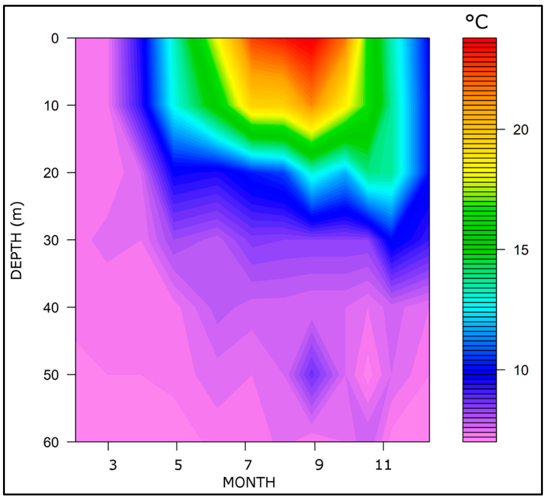

Figure 2. Heatmap of water temperature in Lake Iseo from January to December 2016.

The warmest temperature values were detected in the first 10–20 m during summer, especially in July and August. Below a depth of 60 m, the water temperature was quite constant (6.8 ± 0.7 °C (±SEM)). The lowest water temperature difference in the water column was detected in January and February, with a delta from the top (0 m) to the bottom (245 m) of 0.80 °C. In this period, we assumed water mixing only occurred up to a depth of 50 m by analyzing the chemical parameters along the water column. Starting mid-March, we observed a progressively more pronounced difference in the water column temperature among the superficial and the deeper layers, with an enhanced stratification from May to September. The depth of the thermocline (z_{therm}) ranged from 13 to 15 m.

During 2016, the value of water transparency in Lake Iseo ranged from 2.4 (June) to 10 m (January). In general terms, a greater transparency was observed during the winter months due to the lower primary production compared to summer months. The euphotic depth, ranging between 9 (June) and 23 m (January), was attained using the Secchi disk (z_s). The surface radiation had values of between 40 and 293 W/m^2, with an extinction coefficient in the range of 0.2 to 0.5.

The Brunt-Väisälä frequency (STABILITY) ranged between -6.7×10^{-5} and 1.43×10^{-3} s^{-2}. The values detected at the thermocline ranged between 1.5×10^{-5} and 1.4×10^{-3} s^{-2} and reached maximum values during the summer months, whereas the minimum values were observed during winter [20,54].

3.2. Chemical Characteristics

Dissolved oxygen (DO) was higher in shallower layers. In Lake Iseo, below a depth of 50 m, the oxygen concentration was always critical, due to the high lake productivity, as well as the diminished frequency of deep circulation recorded in recent years. During February and March 2016, a dissolved oxygen recharge in the 40–60 m layers was observed, with a rapid subsequent decrease. During August and September, the metalimnetic oxygen concentration was higher than the levels in the hypolimnion and epilimnion, displaying a positive heterograde curve (Figure 3a).

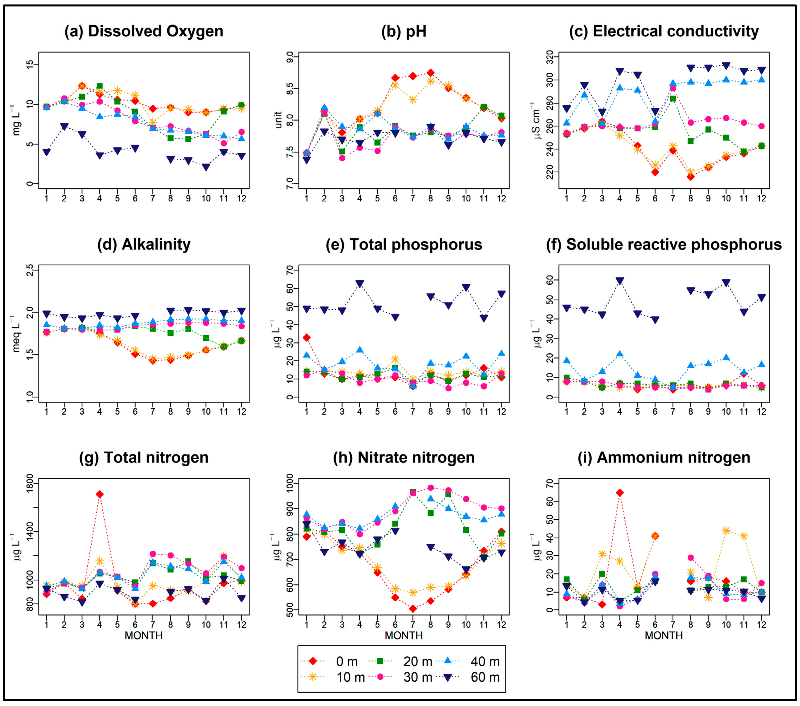

Figure 3. Temporal development of the principal chemico-physical features analyzed at six depths: (**a**) Dissolved oxygen, DO (mg/L); (**b**) pH; (**c**) Electrical conductivity, EC (µS/cm); (**d**) Alkalinity, ALK (meq/L); (**e**) Total phosphorus, TP (µg/L); (**f**) Soluble reactive phosphorus, SRP (µg/L); (**g**) Total nitrogen, TN (µg/L); (**h**) Nitrate nitrogen, NO_3^- (µg/L); (**i**) Ammonium nitrogen, NH_4^+ (µg/L).

pH, measured at 20 °C, showed a similar pattern as oxygen, with a higher value in the superficial layers, where the photosynthetic activity of autotrophic organisms is concentrated, and lower values in deeper layers, where heterotrophs produce carbon dioxide (CO_2), moving the pH toward neutral values. pH values of between 7.1 and 8.7 were recorded, with higher superficial values in June, July, and August, and with the lowest value being recorded in January (Figure 3b).

During the whole period analyzed, alkalinity (ALK) and electric conductivity (EC) were in the range of 1.4 to 2.3 meq/L and 216 to 320 µS/L, respectively (Figure 3c,d).

Total phosphorus (TP) and soluble reactive phosphorus (SRP) showed a similar trend. In epilimnetic layers, the concentration was 12.9 ± 0.7 µg/L and 6.33 ± 0.28 µg/L, respectively for TP and SRP. At a depth of 60 m, where the primary production is low and the microbial loop is well developed, the concentration was 51.9 ± 1.9 µg/L for TP and 49.0 ± 2.1 µg/L for SRP (Figure 3e,f).

In the first 60 m, total nitrogen (TN) was between 800 and 1713 µg/L, with the peak value topside in April and the lowest value in June at 0 m (Figure 3g). The maximum mean value along the water column, from the top to a depth of 60 m, was recorded in April (1153 µg/L), whereas the minimum value was recorded in June (887 µg/L). Nitrogen nitrate ($N\text{-}NO_3^-$) showed lower values at 0 and 10 m, whereas the greatest values were detected at 30 and 40 m, within the range of 505 to 934 µg/L (Figure 3h). Nitrogen ammonium ($N\text{-}NH_4^+$) ranged between 2 and 65 µg/L, with the maximum value recorded in April at 0 m and the minimum value also in April at 30 m (Figure 3i).

Silicates had values between 56 and 1524 µg/L, with the greatest value recorded at 60 m. The silicate concentrations did not differ between the various sampling months, remaining quite constant. Chloride, sodium, and potassium ions displayed similar values throughout the year and along the water column, with values of 3.07 ± 0.02, 2.81 ± 0.01, and 1.29 ± 0.01 mg/L, respectively. For magnesium and sulphates, with respective mean values of 7.45 ± 0.35 and 18.5 ± 0.2 mg/L, a variation in seasonal and spatial values was not observed. Calcium ions showed similar values in the different months at various depths, with a mean value of 41.8 ± 0.39 mg/L, although a slight decrease in the superficial layers (i.e., 0–10 m) was seen from May to October (35.3 ± 0.53 mg/L).

3.3. Seasonal and Spatial Dynamics of *P. rubescens* and *T. bourrellyi*

During the observation period of January to December 2016, both Cyanobacteria were always present in the lake. The mean species biovolume (from top to 60 m) reached its maximum in April, with a value of 585 ± 21 mm^3/m^3 for *P. rubescens* and 162 ± 72 mm^3/m^3 for *T. bourrellyi*. The minimum mean biovolume was recorded in September, with a value of 1.8 ± 0.7 mm^3/m^3 for *P. rubescens* and 4.9 ± 1.5 mm^3/m^3 for *T. bourrellyi* (Figure 4).

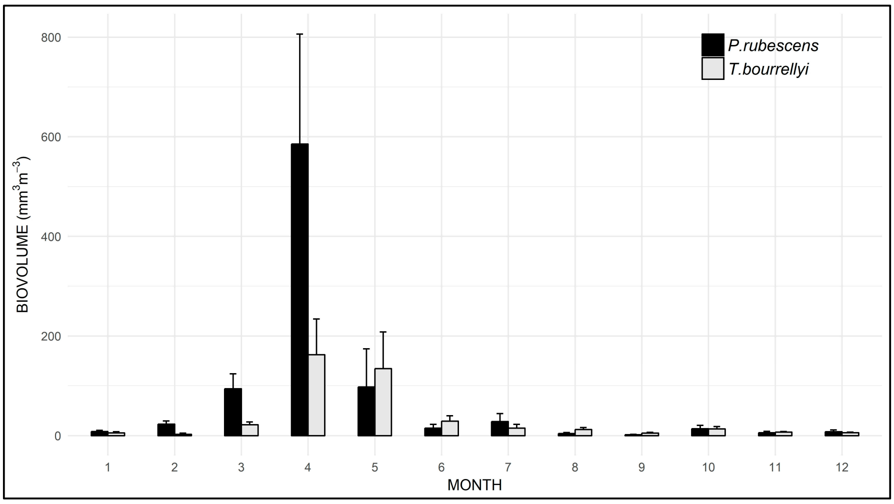

Figure 4. Monthly mean biovolume ± SEM (mm^3/m^3) of *P. rubescens* and *T. bourrellyi* in 2016.

T. bourrellyi started to increase in March and April, whereas a greater abundance of *P. rubescens* was detectable from the first months of the year, i.e., January and February. A temporal shift in the development of the two species can be highlighted. In January and February, the *P. rubescens* abundance had average values of 8.28 ± 2.27 mm^3/m^3 and 22.7 ± 6.35 mm^3/m^3, respectively; whereas *T. bourrellyi* recorded values of 5.6 ± 2.09 mm^3/m^3 and 2.78 ± 2.33 mm^3/m^3. An increase in *T. bourrellyi* biovolume was detected in March, in which a value of 21.5 ± 5.8 mm^3/m^3 was recorded, but the greatest values were detected during the next months, especially in April and May (134 ± 74 mm^3/m^3). The largest *P. rubescens* biovolumes were measured during March (93.9 ± 29.4 mm^3/m^3), and April (585 ± 21 mm^3/m^3). A greater biovolume of *P. rubescens* than that of *T. bourrellyi* was recorded for six months during the observation period: January, February, March, July, October, and December.

The variation of the biovolume of the two Cyanobacteria in the different months was strongly influenced both by cell density and cell volume. The species did not show differences in cell dimension at the different depths along the water column, but seasonal variations were observed. In April, *P. rubescens* considerably increased in biovolume compared to *T. bourrellyi*, as its cells displayed the highest diameter (6.33 µm), with a cell volume of 150 µm^3. The average annual cell volume of *P. rubescens* and *T. bourrellyi* had the same value (63 ± 4 and 63 ± 2 µm^3 respectively), with a mean annual diameter of 4.38 ± 0.11 and 4.2 ± 0.08 µm, and a mean annual length of 3.95 ± 0.04 and 4.50 ± 0.08 µm, respectively. For *P. rubescens*, the maximum cell volume was measured in April, whereas the smallest value was recorded in December (25 µm^3). *T. bourrellyi* showed a maximum cell volume in October (94 µm^3) and the minimum in April (38 µm^3). The surface-to-volume ratio of the two species showed a similar monthly trend, with an average annual value of 1.47 and 1.43 for *P. rubescens* and *T. bourrellyi* respectively. The greatest difference between the Cyanobacteria was detected in April and January. In general terms, *P. rubescens* showed a greater annual variation in this ratio compared to *T. bourrellyi*.

The two species were widespread in the entire sampling zone, and their spatial distribution varied among the different months, with considerable concentrations in the sub-superficial layers. In considering the months with major abundance, *P. rubescens* showed the greatest biovolume around a depth of 20 m, with a relevant, although lower, presence at 10 and 30 m. *T. bourrellyi* displayed a considerable biovolume at depths of 10 and 20 m. Beyond the 40 m depth, *T. bourrellyi* abundance throughout the year was poor, whereas *P. rubescens* was present in considerable amounts at these depths, especially in the months of greater development (March, April, May) (Figure 5).

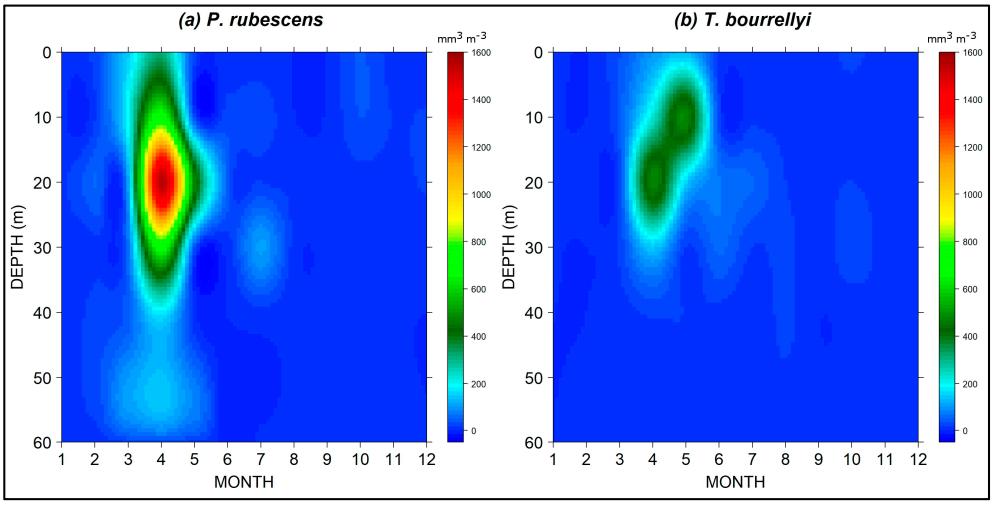

Figure 5. Contour plots showing the spatial and temporal distribution in Lake Iseo during 2016 of (**a**) *P. rubescens* biovolume (mm^3/m^3); (**b**) *T. bourrellyi* biovolume (mm^3/m^3).

3.4. Ecological Requirements of *P. rubescens* and *T. bourrellyi*

To characterize the ecological requirements of *P. rubescens* and *T. bourrellyi*, we performed principal component analysis (PCA), considering the 20 chemico-physical parameters (listed previously) and the biovolume of *P. rubescens* and *T. bourrellyi*. Below, we report only the most meaningful analysis, which includes the preliminary principal component analysis and the final analysis with properly selected observations.

The preliminary PCA showed that the eigenvalues of the first two principal components (PCs) represented 63.4% of the total variance of the observations (PC1 47.6%; PC2 15.8%) (Figure 6a).

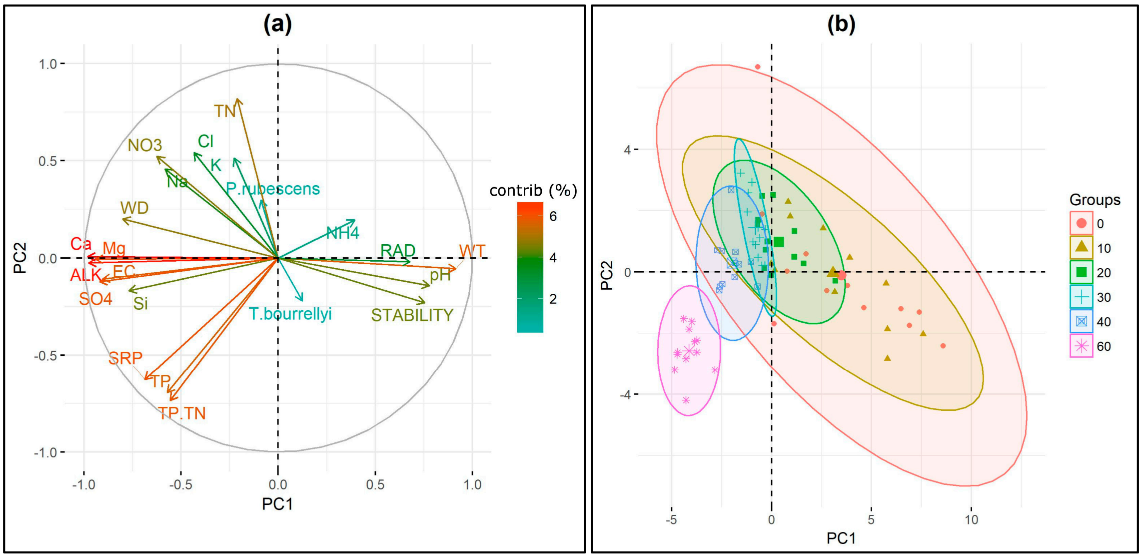

Figure 6. Preliminary principal component analysis (PCA) performed on biovolume values of *P. rubescens* and *T. bourrellyi* and 20 chemico-physical variables, detected during the whole period analyzed from the surface to a depth of 60 m. (**a**) Loading plot on the plane defined by principal components 1 (PC1) and 2 (PC2), with different colors according to the individuals' contributions ("contrib %"). See "Materials and Methods" for the abbreviations; (**b**) Score plot on the plane defined by PC1 and PC2; samples are grouped ("Groups") by different depths in ellipses (confidence level 0.95).

The first factor had the highest positive loadings for water temperature, pH, stability, and radiation; whereas the highest negative loadings were found for calcium, alkalinity, magnesium, sulphates, electrical conductivity, water density, and silicates. The second factor had the highest positive loadings for total nitrogen, and the lowest for chloride, potassium, nitrate nitrogen, and sodium; whereas negative loadings were observed for total phosphorus, and the TP:TN ratio. *P. rubescens* and *T. bourrellyi* biovolumes were poorly explained, and they were not correlated in the plane defined by PC1 and PC2. A good correlation was found between *P. rubescens* and total nitrogen; whereas *T. bourrellyi* showed a slight correlation with stability, water temperature, and pH. The score plot showed a clear difference between the observations at various depths (Figure 6b). The samples collected at a depth of 60 m displayed a different pattern, forming a cluster. This different behavior could be explained by this deep layer being the high hypolimnion, in which different conditions were able to be detected from in the superficial layers. The samples collected in April at 0 m were outliers.

Preliminary PCA allowed the identification of variables and observations on which our attention should be focused in order to determine the relationship between Cyanobacteria and environmental features. The final PCA was performed on observations from five sampling depths (excluding data collected at 60 m) over four months (February–May), in which the species started their development and showed the greatest biovolume. The eigenvalues of the first two principal components of the final PCA represented the 62.6% of the total variance, with a value of 42.0% for the first principal component, and 20.6% for the second (Figure 7).

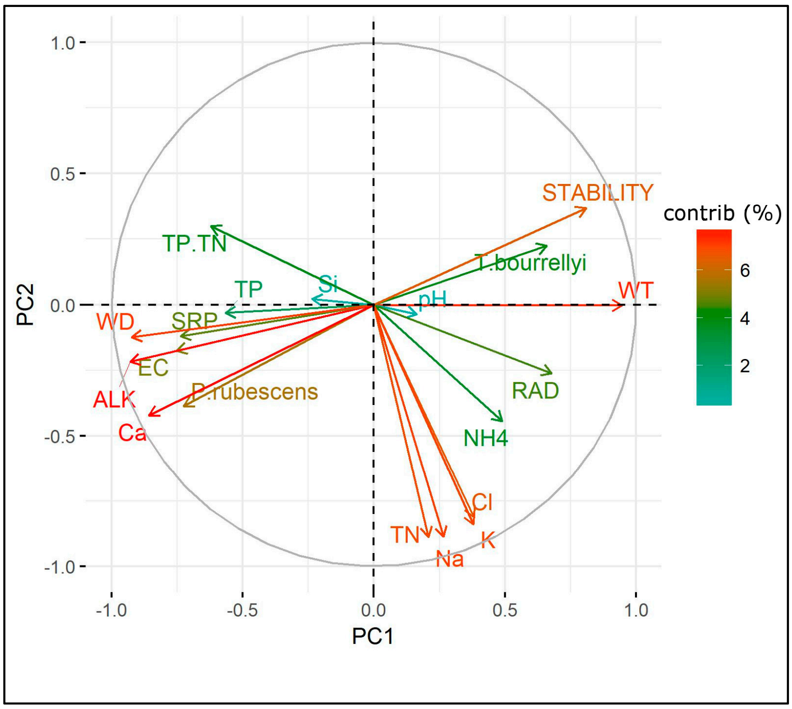

Figure 7. Loading plot of the first two components of the final principal component analysis (PCA) performed on biovolume values of *P. rubescens* and *T. bourrellyi* and 20 chemico-physical variables, detected at depths from 0 to 40 m during February, March, April, and May.

The first component had the highest positive value for water temperature, stability, radiation, and *T. bourrellyi* biovolume; and had the highest negative value for alkalinity, water density, calcium, electrical conductivity, soluble reactive phosphorus, and *P. rubescens* biovolume. Total nitrogen, sodium, potassium, and chloride were explained by the second factor, with negative loading values. A high correlation was highlighted between *T. bourrellyi* and stability, which was linked to water temperature, strengthening the relationship shown above. A correlation was also seen between this species and radiation. *P. rubescens* was highly correlated with calcium, alkalinity, electrical conductivity, and in general with the phosphorus forms.

A linear regression between *T. bourrellyi* and stability was performed to highlight the possible cause-effect relationship. We selected the months in which the species was more abundant, as previously chosen for PCA (i.e., February, March, April, May). The stability significantly and positively affected *T. bourrellyi* biovolume (F = 12.4, $p < 0.002$, $R^2 = 0.36$).

4. Discussion

A proliferation of Cyanobacteria has been recorded in lacustrine phytoplanktonic communities, and studies have highlighted the possible role of climate change in promoting toxic blooms, although considerably different responses to temperature increase have been recorded among various Cyanobacteria species and among genetic strains [8,9,12,55–60]. Moreover, these changes in lake ecosystems can lead to the introduction of allochthonous populations of Cyanobacteria, with different and not-always-identified toxicological features [4,55]. Although these complex events cannot be explained by a single environmental driver [55,61], more effort is needed to further the knowledge about the abiotic and biotic elements that promote the development of harmful Cyanobacteria.

The phytoplanktonic communities in the deep south-Alpine lakes in Italy have been repeatedly monitored and studied. A long-term data set (from 1998 to 2016) was available for Lake Iseo [21,22]. Starting in 2014, the presence of the allochthonous *T. bourrellyi* (Cyanobacteria) was detected in Lake Iseo, as well as in other south-Alpine lakes. The causes of its proliferation remain as yet poorly understood [3,4].

Our study found high biovolume values of *T. bourrellyi* in Lake Iseo, with a maximum abundance of 162 mm^3/m^3. These recorded values were comparable to those of *P. rubescens*, which was reported in previous studies as the most abundant Cyanobacteria taxa in Lake Iseo [21,22]. These two species (order Oscillatoriales) are multicellular filamentous forms, cold-stenotherm species, with a comparable growth temperature range of 10 to 20 °C [62]. A remarkable difference between the two species is related to the gas vesicles. Unlike *T. bourrellyi*, *P. rubescens* has these structures and, therefore, has an important competitive advantage. *P. rubescens* is able to move along the water column and consequently acquire the most favorable position [4,63–67]. The physiological features and environmental factors that allow the co-presence of these two Cyanobacteria have not yet been identified. In particular, understanding which environmental changes have occurred, and on what timescale, is important, as these changes did not sufficiently favor *P. rubescens* for *T. bourrellyi* to be excluded, thus permitting their co-existence [68,69].

In 2015, a stronger development of *T. bourrellyi* compared to *P. rubescens* was recorded in Lake Garda. The re-oligotrophication process of this lake has been hypothesized to be a possible explanation for the altered cyanobacterial community [4]. In the last decades of the 20th century, a decline in the *P. rubescens* population was recorded in many peri-alpine lakes, which was linked to the decrease in nutrients loading following large restoration projects, e.g., Lake Garda, Lake Maggiore [31], Lake Bourget [70], and Lake Ammersee [71]. This situation was not the case for Lake Iseo, in which nutrients have been high since the 1970s [21,22]. Moreover, for the different lakes, phytoplankton composition is largely dependent on their having similar geographic, morphometric, and hydrodynamic characteristics, whereas the biomass level is mainly determined by nutrient load [23]. The role of temperature and nutrients in affecting *P. rubescens* development is still open to considerable debate [20,72,73]: in oligotrophic lakes, the nutrients appear to be more important than the temperature; in mesotrophic lakes the temperature has a stronger impact; and in eutrophic and hyper-eutrophic lakes, the positive interaction between temperature and nutrients plays the main role [74].

On a global scale, an increasing trend in the lacustrine water temperature has been widely reported, with a mean rise of 0.34 °C/decade in the summer surface water temperature [75–77]. In particular, studies on Lake Iseo highlighted an average annual increase of 0.19 °C/decade, with a temperature rise of 0.56 °C in the 30 years from 1986 to 2015 [77]. Rising temperatures could affect the pelagic community, by both directly and indirectly influencing the growth, the survival, the metabolism, and the reproduction of organisms [8]. One of the most important consequences of this temperature increase is stronger thermal stratification, which is caused by the temperature-dependent density difference between the epilimnion and hypolimnion [78], as well as the decrease in the intensity of deep mixing, which has been observed in Lake Iseo since the mid-1980s [25]. The most recent episodes of full circulation occurred at the end of winter in 2005 and 2006, and were due to extremely cold winters and sufficient kinetic energy [35,36,79]. From 2007 until 2014, the mixing depths likely did not exceed 100 m, and the most recent years (2014–2016) have been characterized by a mixing depth accounting for no more than 20% of the water column [35,36,80]. By analyzing the available discrete data for 2016, we detected a maximum deep water mixing of 50 m.

The presence of *P. rubescens* in Lake Iseo has been reported since at least the 1980s [21,22]. The presence of gas vesicles in this species could allow the overcoming of the tendency of Cyanobacteria to sink out of the euphotic zone, providing *P. rubescens* with a competitive advantage. Changes in cellular weight due to photosynthesis and respiration processes can be balanced with gas vesicles [65,66,81]. Moreover, the buoyancy ability allows each species to optimize light absorption and obtain the most favorable positioning in the water column. This species, being tolerant to light deficiency, tends to form a layer on the lower border of the metalimnion in deep and stratified lakes, where it can avoid interspecific competition and exploit resources and nutrients [65,74,82]. The gas vesicles can, however, irreversibly collapse at a certain critical hydrostatic pressure and then no longer provide buoyancy. Consequently, the depth to which the planktonic organisms can be mixed in winter is of paramount importance, determining the amount of Cyanobacteria that remain vital at the end of the winter and that form the inoculum for growth during the next season [23,83]. D'Alelio et al. [84] demonstrated that the presence of weaker or stronger gas vesicles in *P. rubescens* is an adaptive based on shallow or deep winter circulation, respectively. In 2009 in Lake Iseo they also underlined the presence of *P. rubescens* strains with strong gas vesicles and a critical collapse pressure (p_c) of $\cong$1.1 MPa that were able to overcome hydrostatic pressure of water depths of up to 80–100 m in winter. Given the reduced mixing depth recorded in Lake Iseo in recent decades, *P. rubescens* was able to maintain a reproductive population during the winter months with weak vertical circulation, i.e., January and February. For this reason, and given the ability of *P. rubescens* to use limited solar radiation, this population was able to grow early in the first months of the year, gaining a competitive advantage over other phytoplanktonic taxa, e.g., *T. bourrellyi*.

T. bourrellyi does not possess gas vesicles, and, during calm, thermally stratified conditions, sinks to form a metalimnetic maximum [67]. As this species does not have a buoyancy ability, a different strategy is used to optimize light absorption. These features make this species well-adapted to stratified lakes [67]. A deep-water mixing would bring this organism out of the euphotic zone, where the conditions are not suitable for its survival. Our PCA analyses are in agreement with this statement, showing a strong relationship between *T. bourrellyi* and the parameters of stability (Brunt-Väisälä frequency) and radiation. Then, when the stratification was more obvious and the radiation higher, the species began its development. The nutrients, instead, seemed to be a less critical factor in promoting one species over the other.

Despite the shift in spring development, both Cyanobacteria attained maximum biovolume in April; the biovolume then decreased from June to January. The same temporal development of *T. bourrellyi* was highlighted in Lake Garda (deep south-Alpine lake) in 2015, with the maximum reached during April and May, and a remarkable decrease taking place in August [4], confirming that the most suitable conditions for this species occurred during the spring months. The extremely high biovolume of *P. rubescens* in April was related to the elevated cell volume, especially cell width, as the densities of the two Cyanobacteria were comparable. At present, clearly evaluating what adaptive significance the variation in cellular dimensions could have is not possible. We only speculated that the greater cellular dimension observed could be related to the increase in gas vesicle diameters. In April, a more efficient buoyancy capacity, proportional to vesicle volume [85], could favor the movement of the organism in the stable water column. In our work, gas vesicle diameter measurement was not performed. In the literature, to the best of our knowledge, no one has verified this hypothesis. However, the inverse relationship between the gas vesicle width and the critical pressure at which they collapse has been documented [85,86]. Therefore, *P. rubescens*, during the mixing period in which the strains could be carried at depths with elevated hydrostatic pressure, developed smaller and stronger gas vesicles, to avoid their collapse. The enhanced stability condition allowed the increase of the gas vesicles diameter and consequently the movement of the organisms became more efficient. The increase in cellular dimension involved a change in the surface to volume ratio, influencing nutrients and light absorption [87]. With the increased cell width, the ratio became unfavorable in April for *P. rubescens*.

P. rubescens attained a maximum biovolume around a depth of 20 m; *T. bourrellyi* showed a different pattern with a spatial shift from April (20 m) to May (10 m). This behavior could be linked to the upward repositioning of the thermocline, detected from April (z_{therm} = 15.7 m) to May (z_{therm} = 13.1 m) (Figures 2 and 5). The vertical distribution of *T. bourrellyi* was tied to the thermocline positioning in the water column, as this species does not possess gas vesicles and cannot independently move in the water column, and accumulated in alignment with the major density-temperature discontinuity [4,67]. The presence of gas vesicles in *P. rubescens* allowed this species to move along the water column and grow in the layer with the best irradiance conditions, which were detected in Lake Iseo at a depth of around 20 m. This species is also well adapted to low light values, and has been reported to be an efficient light harvester [65,81,88]. It was detected growing in the radiation conditions between 40 and 60 m, where it developed with lesser biovolume values than in the upper layers.

In conclusion, this work assessed the environmental changes that have occurred in Lake Iseo that permitted the settlement of the allochthonous Cyanobacteria *T. bourrellyi*, and the co-existence with *P. rubescens*, which still had a large biovolume. Our results confirmed that *P. rubescens* was competitive not only in stratified lakes, but also in lacustrine ecosystems characterized by partial mixing in the water column. Its survival was due to its features; *P. rubescens* was able to survive the winter, and started to grow immediately when allowed by the radiation and stability conditions. The increased stratified conditions in the aquatic system seemed to be a key factor for the settling of *T. bourrellyi*, which cannot proliferate in lakes with deep mixing events. These results suggest the possible role of enhanced stability in promoting the development of this allochthonous Cyanobacteria, which has created its own niche as a result of the more stable water column and reduced mixing depths in the winter. Climate changes can directly and indirectly affect aquatic environments, and more effort is needed to better understand the modified dynamics in lakes, and to expand the knowledge obtained by annual investigation, to understand if what was verified in Lake Iseo could be found in the other southern Alpine lakes.

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